Chapter 7

7 General discussion and outlook

Irshad Ul Haq

The soil environment as a matrix for microbial life

Structurally, soil is an environment that developed to its current form of solids and pores as a result of mechanical, physical, chemical, and biological processes that acted together. Soil therefore varies spatially and temporally, making it a complex environment for the inhabiting microbiota (Standing and Killham 2007). This concerns in particular the local soil bacteria, in their efforts to reach resources. Basically, soil bacteria are confined to the microhabitats they inhabit, and their exploration of other microsites, even when nearby, is often spatially–limited by physical barriers that limits their migration. A second key issue is the general recalcitrancy of carbonaceous soil substrates to degradation. The two phenomena lead to a generic scarcity of easily–available and degradable C (and energy) sources for heterotrophic bacteria. This results in (average) slow or arrested bacterial growth and survival of cells in any state of 'dormancy'. Other factors that – directly or indirectly – exert pressure on the soil bacteria is the water potential of soil. The water potential, which is 'inversely congruent' with the water level in soil, is key to the motility of bacterial cells (both passive and active), the diffusion of nutrients, pH, (buffering of) temperature and aeration. Regarding the latter, the activity of microbes in soil is clearly determined by the soil's redox potential, which has a strong relationship with the local availability of oxygen. However, according to Standing and Killham (2007), soil–microbe relationships are not simply one–way, but they should be looked at as a two–way process, in which "the soil shapes the microbes and the microbial activity shapes the soil habitats". I here posit that the major challenge to bacterial life in soil is the necessity to be vigilant to adverse conditions, and thus the 'holy grail' of soil bacteria is their key ability to (continuously) monitor, explore and adapt to the local soil conditions.

Soil habitats and the role of fungi in supporting bacterial life

The living soil encompasses a system hosting a plethora of life forms, which – next to soil bacteria – also includes Eukarya such as plants and fungi. Whereas plants are clearly important primary producers, soil fungi are considered to be extremely important biological forms that are involved in major processes of organic matter decomposition and mineralization (Finlay 2007). A particular feature of the soil fungi is their capacity to cross the physical barriers of soil, mainly the air gaps that form barriers to the soil connectivity. Such gaps prevent even motile bacteria from moving far in the soil matrix. In effect, the soil fungi are able to form a connected matrix of hyphae (hyphal network), establishing bridges over the disconnected microsites (Kohlmeier et al. 2005). This property makes them very successful in exploring niches in soil, such as those under the influence of plant roots – the rhizosphere. As brought up in the introduction to this thesis, the interconnectivity of plant roots and the hyphae of plant–root–associated fungi (mycorrhizal fungi) results in a habitat that is under the combined influence of fungi and plants [denoted the mycorrhizosphere]. An enhanced microbial activity is expected to occur in this sphere (De Boer et al. 2005). Next to the mycorrhizosphere, other, rather

transient, zones of microbial activity occur at fungal hyphae such as under mushroom feet or directly at the hyphal network [denoted the mycosphere].

Given their ability to connect soil microsites, next to their capacity to spur microbial activity as a result of the provision of resources, fungi can be seen as key 'rescuers' of soil bacteria in terms of the ecological opportunities they appear to offer. For instance, some bacteria may actually rely to a large extent on their ability to establish relationships with soil fungi, in order to explore the hospitable microhabitats in the soil that are beyond their reach without a hyphal network. Such a capacity may support their survival and dispersal over large patches of area in the soil and not just confined to a ~5– m soil aggregate located microsite. Thus, for particular heterotrophic soil bacteria, the answer to C starvation, with respect to survival, dispersal and sustainability in soil, may well lie in their interactive capabilities with divergent soil partners, including fungi (as well as with plants).

The complexity of bacterial–fungal interactions in soil and the efforts to understand them

The field of bacterial–fungal interactions (BFI) is relatively nascent in terms of the understanding of the underlying principles. However, as I discussed in the previous chapters of this thesis, in particular **chapter 2**, key assets of the BFI in soil have already been pinpointed. For instance, several fungal–associated bacteria have been shown to benefit from the ecological conditions offered by their fungal hosts, thus establishing successful symbiotic relationships.

In fact, much of the earlier work in this area has highlighted several *Burkholderia* species as prime occupants of the mycospheres of diverse soil fungi and even as endofungal occupants (Warmink and van Elsas 2008, Stopnisek et al. 2015, Partida–Martinez et al. 2007). In particular the species *B. terrae* (strains BS001, BS110, BS007, and BS437), *B. phytofirmans* (strains BS455, BS421 and BS413), *B. caribiensis* (BS442), *B. xenovorans* (BS416) and *B. terricola* BS454 (Nazir et al. 2012; note that the taxonomic information is – for some strains – not final) were found to be fungal–interactive based on their 'single–strain' migratory capacity along with the hyphae of *Lyophyllum* sp. strain Karsten. Of these, *B. terrae*, being an aerobic, heterotrophic and mesophilic soil dweller, utilizes a broad range of C compounds (Yang et al. 2006); on the basis of its capacity to fix nitrogen, it might constitute an excellent partner for some soil–dwelling fungi. Nazir et al. (2012) also reported on the ability of strain BS001 (and some other strains) to use a wide range of sugars, organic acid and sugar alcohols.

On the basis of the capacities of *B. terrae* BS001 and related strains for life in the mycosphere, I selected these strains and here addressed issues of the interactivity of *B. terrae* (in particular strain BS001) with selected soil fungi. Concerning the latter, *Lyophyllum* sp. strain Karsten and, in a later stage, *Trichoderma asperellum* 302 were used, as representatives of, respectively, the basidiomycota and the ascomycota. In effect, *Lyophyllum* sp. strain Karsten was selected because it was used as a 'model' organism for saprotrophic soil–exploratory fungi and *T. asperellum* 302 was selected because strain

BS001 successfully migrated with it (Nazir et al. 2014). As outlined in the introduction, some of the outstanding research questions pertaining to the ecology and mechanisms of the interactions were answered, as follows:

1. What are the genome size and composition of *B. terrae* BS001 and to what extent do the genes and operons underpin the interactivity of BS001 with diverse fungi in soil?
2. What genes and operons of *B. terrae* BS001 are transcribed and thus important for its survival and interaction with *Lyophyllum* sp. strain Karsten at different time points during the interaction in soil?
3. How do partners in the bacterial–fungal interaction sense the presence of each other? And, are there any communication channels between the two partners?
4. What potential anchoring sites do fungi possess on their cell walls that fungal–interactive *Burkholderia* species attach to?
5. To what extent are fungal–interactive soil *Burkholderia* species attracted towards fungal–released compounds?
6. What is the role of oxalic acid/oxalate in the interaction between *Burkholderia* species and soil fungi?

These specific questions are addressed in the sections below and the data obtained are interpreted in a broader context.

Theoretical mechanisms of interaction between soil bacteria and fungi: with an emphasis on *B. terrae* BS001

In **chapter 2**, I address how the predominant bacterial types that are associated with different fungi may behave in the mycosphere, mycorrhizosphere and in the soil. I provide a detailed overview of the developing concepts of BFI in soil, with emphasis on *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten. These bacterial–fungal as well as bacterial–fungal–plant interactions in soil are argued to play crucial ecological roles (transportation of bacteria along fungal hyphae, influxes of C compounds in their respective zones of influence, protection against antagonists and survival) that influence the ecophysiologies of all the partners. The considerations then zoomed in on the *L. proxima* and *Lyophyllum* sp. strain Karsten mycospheres, where the interaction of *B. terrae* BS001 with the fungal hosts occurs (Warmink and van Elsas 2008, Warmink and van Elsas 2009). A key argument provided was the importance of understanding the genome of strain BS001, as a token of past adaptive processes to the mycosphere and possibly other systems. Overall, the hypothetical concepts forwarded in this chapter provided the basis for the experimental work carried out in the following chapters.

The genome of *B. terrae* BS001 – a wealth of genetic information

For any organism to adapt to, and occupy, spatially and temporally different soil microhabitats, it is imperative to possess the genetic capacity that allows flexibility in adaptive processes to such microhabitats. Konstantinidis and Tiedje (2004) first indicated that

bacteria with larger genomes are ecologically more successful in soil, where 'nutritional scarcity exists and the penalty for slow growth is lower'. At the onset of my work, there were indications that the genome of *B. terrae* BS001 is extremely large, and so one may posit that the bacterium has adapted to a whole suite of conditions that naturally occur in the soil. This is consistent with tenets by Warmink and van Elsas (2009), who stated that *B. terrae* BS001 – although isolated as a typical mycosphere dweller – may actually well have the ability to adapt to different niches in soil (Warmink and van Elsas 2009). Nazir et al. (2012) later showed that *B. terrae* BS001 is able to utilize a wealth of carbonaceous compounds, including citric acid, acetic acid and formic acid, which further supported the metabolic versatility of the organism. In order to unravel the genetic potential of strain BS001, I performed a detailed analysis of its genome, in a comparative genomics study (using 23 *Burkholderia* genomes; **chapter 3**). This included an analysis of past horizontal gene transfer events. The exceptionally large genome of *B. terrae* BS001 (11.5 Mb) contains a range of genetic systems, including those involved in motility and chemotaxis, nutrient sensing/acquisition/transformations, stress responses, protein secretion, biofilm formation and secondary metabolite production, that may confer mycosphere competence to the organism. From the genomics data, I surmised that these systems indeed can provide selective advantages, in terms of spurring survival, dispersal and metabolic rearing, to strain BS001 in the mycosphere as well as beyond.

In a previous study, a preliminary tree, showing the phylogenetic neighbors of strain BS001, was shown (Nazir et al. 2012; based on partial 16S rRNA sequences). Here, I extended this preliminary analyses by making use of seven concatenated core genes (*aroE*, *dnaE*, *groeL*, *gyrB*, *mutL*, *recA* and *rpoB*) to produce a more robust phylogeny. On the basis of this novel information, I inferred that *B. terrae* BS001 belongs to the so–called non–pathogenic clade of the genus *Burkholderia*. The findings are in accordance with previous data, where the concept of pathogenic and non–pathogenic clades was previously reported (Estrada–de los Santos et al. 2013).

In particular, **chapter 3** placed emphasis on the type 3 secretion system (T3SS), as well as other protein secretion systems (T2SS, T4SS and T6SS), presumed to be important for the successful interaction of the *Burkholderia* with soil fungi. As such, I performed comparisons with such systems in other related *Burkholderia* species, including the endofungal *B. rhizoxinica* HKI454. The analyses indeed confirmed the presence of protein secretion systems of types 3, 4, 6 (**chapter 3**) in the genome of *B. terrae* BS001, as well as in other genomes within this genus.

Considering its evolutionary history, *B. terrae* BS001 may have dwelled, over time, in a suite of (geographically distinct) niches, and so established an "evolutionary metacommunity" connected through time (Leibold et al. 2004). Warmink and van Elsas (2009) reported, as a key asset, the migration of *B. terrae* BS001 along the hyphae of *Lyophyllum* sp. strain Karsten in soil. This allows to infer that strain BS001 is adapted to this mycosphere, or, in a broader perspective, these adaptations may likely be extendable to other systems that offer similar conditions. Indeed, the large genome size of strain BS001 is consistent with the tenet that the organism may even have acquired capacities

allowing multifaceted life style phases in the soil. I thus surmised that horizontal gene transfer (HGT) events have played a large role over the evolutionary trajectory of strain BS001. Indeed, evidence was found for such HGT events, as a large portion of the strain BS001 genome contained regions of genomic plasticity (RGP). Such transferred (blocks of) genes may have been selected by past niche conditions, and the fact that we still find them pleads against strong forces of de–selection. Consistent with the multi–niche hypothesis is the fact that *B. terrae* BS001 was able to become associated with a high number of fungal species (Nazir et al. 2014). Interestingly, one of the RGPs (RGP76, 70.42 Kb) harboured a T4SS and other plasmid–related genes i.e. *mobB*, *mobC*, *repB*, *korC*, *klcA*, *parA*, *parB* and *parD* (**chapter 3**), possibly indicating that HGT events may have taken place with this potential vector.

The aerobic heterotroph *B. terrae* – for its survival and growth – requires carbonaceous compounds such as sugars, sugar alcohols and organic acids. Strain BS001 has apparently invested strongly in the capacities to sense, capture and utilize a wide range of carbonaceous compounds, as large numbers of CDSs (11.1% of the genome; **chapter 3**) encode various membrane transporters with presumed involvement in such processes. The consequent large genome size, reflecting a wide range of metabolic as well as stress tolerance mechanisms, is consistent with the theory of Konstantinidis and Tiedje (2004), who showed that larger bacterial genomes tend to accumulate genes involved in regulation, secondary metabolism and energy conversion. Metabolism and compound transport–related genes tended to increase in numbers with increases in genome size (more than 15%; Konstantinidis and Tiedje 2004). Connected to the consequent metabolic activity spurs, chemically–mediated antibiosis is often prevalent. In such interaction, the release of antibiotics or other products (secondary metabolites) by microorganisms is aimed at countering other microbes. In **chapter 3**, I describe the presence of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters, highlighting the potential of strain BS001 to engage in such biochemical warfare. Although I did not identify the potential products of such clusters, I here postulate that these will have significant roles in the ecology of strain BS001 in soil. Interestingly, I found a varied expression pattern of the PKS/NRPS in the transcriptome analyses (see next section), where at the early 'non–physical–contact' stage the expression was downregulated contrary to an upregulation at the physical–contact stage (**chapter 4**).

B. terrae BS001 reveals differential gene expression during the interaction with *Lyophyllum* sp. strain Karsten

In **chapter 4** I investigated the transcriptional responses of *B. terrae* BS001 in the presence versus absence of *Lyophyllum* sp. strain Karsten. I hypothesized that some genetic systems of strain BS001 might respond dynamically, in a time–dependent fashion (physical and no–physical contact), to *Lyophyllum* sp. strain Karsten upon confrontation. I addressed these questions through RNA sequencing using a soil extract agar (SEA) system that was purposefully devoid of easily accessible carbon sources. The resulting data

hinted at a great complexity, and spatiotemporal variability, of the strain BS001 gene expression events that took place. Temporally speaking, there was a clear indication of the occurrence of chemotaxis and signalling (communication) at the onset of the interaction, when both counterparts were physically apart from each other (**chapter 4**). However, the conditions on the SEA plates did not allow the visualization of the taxis (due to a high agar level).

Furthermore, the use of SEA devoid of added key carbonaceous nutrients strongly impacted the dynamics of the bacterial–fungal interaction. A prime observation during this study was the overwhelming expression of stress response related genes, i.e. *rpoS*, *dnaK*, *recA*, *groEL*, *ftsZ*, *mutL* and *mutS*. This observation reflected the [mimicked] nutritionally–scarce soil conditions. Stopnisek et al. (2015) suggested that, during the interaction of *Burkholderia glathei* with *Alternaria alternata* and *Fusarium solani*, the fungi could alleviate the conditions of nutrients scarcity, however during this process, they also caused oxidative stress responses in the bacterium. I posit here that our data, revealing partial stress alleviation in strain BS001 by *Lyophyllum* sp. strain Karsten, are coupled to metabolic influxes into the bacterium concomitant with oxidative stress–at the physical contact stage. This was evident, as the transcriptional dynamics of the interactome changed when *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten physically came into contact with each other. Specifically, a cluster of five genes (see Fig. 4.5 and Table 7.1), consisting of two operons, showed significantly and strongly upregulated expression

Table 7.1 Genetic systems with their potential roles in the interaction of *Burkholderia terrae* BS001 with *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302.

S.No.	Genetic systems/Ecological behaviour	Potential role/Relevance	References/chapter
1	Chemotaxis and flagellar motility genes and their upregulation	Chemotaxis/communication	This thesis/ 3 and 4
2	Chemotaxis towards fungal hyphae, exudates and oxalate	Niche exploration via signals	This thesis/ 5 and 6
3	Oxalate degradation genetic potential in *B. terrae*	Oxalate utilization	This thesis/ 6
4	Oxalate and other metabolites production by *Lyophyllum* sp. strain Karsten and *T. asperellum* 302	Signalling and rearing	This thesis/ 6
5	Five genes' cluster and short–chain dehydrogenases upregulation	Metabolic influxes/oxidative stress response	This thesis/ 4
6	PKS/NRPS presence, dynamic expression during *B. terrae* interaction with *Lyophyllum* sp. strain Karsten	Secondary metabolism	This thesis/ 3 and 4
7	SET domain containing protein and its upregulation	Possible roles in host physiology modulation	This thesis/ 4
8	Type 6 secretion system enhanced expression under soil mimicking conditions	Potential roles in stress response	This thesis/ 3 and 4
9	Type 4 secretion system and plasmid	Horizontal gene transfer	This thesis/ 3
10	Presence of the *gup* gene and glycerol production by fungi	Horizontal gene transfer and rearing	This thesis/3 and 6

compared to the control treatment. The aforementioned gene cluster was probably dedicated to situations where both the ecological benefits from the degradation of compounds such as oxalate or its complexes (potentially coming from the soil fungi) can be reaped, as well as potential stresses neutralized.

The information gathered in **chapter 4** revealed the high complexity of the expression patterns of *B. terrae* BS001 during its interaction with the fungus in a carbon–limited situation. I argue here that a more hypothesis–driven approach should be applied to understand each one of them in depth.

Does *B. terrae* BS001 swim towards soil fungi and what are the potential anchoring sites for the bacterium on the fungal cell wall?

Active bacterial motility is dependent on mechanisms like swimming, swarming and gliding (next to twitching), and is collective in nature (Ben–Jacob et al. 2016). Despite the fact that each bacterial cell might be equipped with flagella, a concerted movement towards a source or a signal is often seen (Ben–Jacob et al. 2016). From the transcriptome data described in **chapter 4**, and consistent with the theoretical insights stated in **chapter 2**, I derived the hypothesis that the interaction between *B. terrae* BS001 and two soil fungi (*Lyophyllum* sp. strain Karsten and *T. asperellum* 302) is driven by directed taxis towards the fungal hyphae, followed by attachment to fungal surface anchoring sites. Furthermore, the movement (swimming) of *B. terrae* cells towards fungi might be collective in nature, and there might be a communication channel among the bacterial cell population, prior to the decision of movement in a certain direction. Therefore, to understand the spread of BS001 cells in an ecological sense, in **chapter 5** I analyzed the ecophysiological behavior of *B. terrae* BS001 during its interaction with *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. Indeed, positive chemotactic responses were found when both fungi were sensed. This confirmed, first of all, the attractant function of the two soil fungi. Next, it indicated a certain commonality in the responses, reflecting potentially similar signals sent out by the fungal mycelia. Interestingly, the extent of chemotaxis towards the two fungal species was different, which might reflect their different physiological behavior. One line of explanation is that different metabolites are released by the two fungi. Given that *T. asperellum* 302 was a faster grower than *Lyophyllum* sp. strain Karsten, a key driving factor may also be "timing", e.g. of the release of chemoattractants. Different compounds may have occurred at different levels or concentrations in the exudates of the fungi, which formed the gradients. Thus, such chemical cues (Chet and Mitchell 1976) may have varied between the fungal host species, suggesting a somewhat varied response towards these (Zentmyer 1961).

Chapter 5 then explored the effects of compound level on the chemotactic behaviour of strain BS001 towards the selected soil fungal extracts. Previously, glycerol was shown to be a major compound exuded by *Lyophyllum* sp. strain Karsten, at approximately 2 mM. Later, Nazir et al. (2013) also found glycerol is released by the fungus in standing liquid microcosms, into viscous droplets on top of mycelial mats. On the other hand, in

previous analyses oxalate was not measured and hence the potential role of oxalate as a signalling molecule (Rudnick et al. 2015) in the interaction of *B. terrae* and soil fungi was missing. Here, the effects of these two compounds, glycerol and oxalate, on the chemotactic responses of *B. terrae* strains towards two fungal species was assessed. The chemotactic response of strain BS001 increased as the concentration of glycerol in the medium increased. Remarkably, BS001 showed stronger/faster chemotaxis at a lower concentration and a lesser response at higher concentrations of oxalate. In *B. phytofirmans* PsJN mutant Δ*oxc* , the possible toxicity of oxalate was addressed, but the strain did not reveal any significant response (Kost et al. 2014). Hence, the potential role of oxalate as a toxic compound to *Burkholderia* sp. is not fully understood at present. I interpret our collective data of the experiments with glycerol and oxalate, as glycerol providing the energy for positive movement of strain BS001 towards the oxalate source. The latter compound thus acted as signalling molecule at the low concentration (0.1%, equivalent to 7.9 mM). At higher concentrations, the strain BS001 cells may have been 'deceived' by a 'sufficient' supply of carbon and energy, and therefore migrated towards the fungi to a lesser extent. In systems addressing the swarming motility of *Collimonas fungivorans* Ter331, oxalate (50 M) was suggested to also act as a signaling molecule (Rudnick et al. 2015). However, at higher oxalate levels (500 M), the swarming intensity receded and so it was suggested that such swarming may become arrested once the source of the signal is located (Rudnick et al. 2015).

The physical interactions between *B. terrae* BS001 and the two soil fungi were then further explored (**chapter 5**). In a collaborative work with partners in UFRJ, Brazil (group of Prof. Barreto–Bergter), I thus investigated the potential anchoring sites on the fungal cell surfaces and the mechanisms involved in the physical interactions between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. The analyses revealed that the order of colonization (bacteria first and fungus second or *vice versa*) was important, as significantly higher biomass (reflecting the co–culture) was found when bacteria were inoculated first and the *Lyophyllum* sp. strain Karsten as the second colonizer. However, the behaviour of *B. terrae* BS001 towards *T. asperellum* 302 was different, as joint biofilm formation was not easily observed. The attachment of bacterial cells to the fungi was suggested to involve surface–exposed molecules, in particular the glycolipid ceramide monohexosides (CMH). The data suggested that CMH is one of the potential binding sites on the *Lyophyllum* sp. strain Karsten's surface for strain BS001. The binding of strain BS001 to CMH molecules of *T. asperellum* 302 was lower. We hypothesized that a difference in the ceramide portion of the CMH molecules of both fungi, where an additional hydroxyl group at C–4 was present in the CMH of *T. asperellum* 302, might be at the basis of the difference, however this position needs confirmation.

Does oxalate act as a signalling molecule or a potential carbon source?

Oxalate is a highly oxidized compound which contains two carbon atoms with an oxidation number of +3 and only two electrons (Schneider et al. 2012). It is 'generally'

considered to be a 'poor' substrate compared to other carbon sources for soil bacteria such as glucose and glycerol. The precise role of oxalate in the interaction of *B. terrae* BS001 and fungi in soil was still enigmatic, in particular whether the capacity to perceive it as a signal as well as to utilize it, would be shared among different strains. In **chapter 6**, I tested the hypothesis that oxalate may be acting as a signaling molecule for such strains, to sense the presence and location of fungi in soil. I conclude that, at low concentration (0.1% [w/v]), oxalate incited a strong chemotactic response in all *B. terrae* strains, including the type strain 17804^{T}. However, such responses were 'lethargic' at higher concentrations of oxalate. I then looked at the chemotactic responses of all *B. terrae* strains to exudates of both fungal species at different levels. Interestingly, both exudates attracted all *B. terrae* strains when used undiluted. However, this occurred to varied levels by the different strains. Prior information on the use of oxalate by *Burkholderia* was scarce. Clearly, I showed that oxalate–amended M9 medium supported the growth of strain BS001, indicating it was able to use oxalate as the carbon and energy source for its active growth {at 0.1% and 0.5% [w/v]}. This becomes plausible due to the fact that the genomes of strains BS001, next to BS007, BS110 and BS437, harbor genetic systems that are potentially involved in the utilization of oxalate. I here posit that *B. terrae* BS001 is oxalotrophic, yet may use oxalate as a last–resort compound. Dijkhuizen et al. (1978) revealed that *Pseudomonas oxalaticus* (now known as *Cupriavidus oxalaticus*) follows a diauxic growth pattern when the bacterium is provided with a mixture of oxalate and formate in a batch culture, preferring formate as a substrate. This behavior is predictable, as the energetic potential of oxalate is lower than that of formate. Although this thesis did not pursue the potentially diauxic growth patterns of strain BS001, I expect a diauxic growth in cases where both glycerol and oxalate are offered. The conjecture that oxalate may not be a preferred carbon source for the growth of *Burkholderia* is corroborated by the findings of Dijkhuizen et al. (1977), where the active transport of oxalate across the membrane had a large effect on the energy budget of the cell. That is, 50% of the potential energy contained in oxalate was required for its translocation across the cell membrane, compared to 25% for formate. Hence, the energy possessed by oxalate may not be sufficient for the vigorous growth of *B. terrae* strains like BS001. Thus oxalate may be a signalling molecule in 'first place' and a 'potential' carbon source in the second (Rudnick et al. 2015; **chapter 6**). In the mycosphere, *B. terrae* BS001 may perceive released oxalate as a signal to locate the fungal source, and – being equipped with oxalate catabolic genetic systems – may also use it as a 'second–option' carbon source (i.e. following depletion of released glycerol) in the nutrient–depleted microenvironment.

Do different fungal types provide similar or different ecological opportunities to *Burkholderia terrae* strains?

Although Boersma et al. (2012) described the composition of exudates released by *Lyophyllum* sp. strain Karsten, evidence for the presence of oxalate has been lacking due to the method used for identification (^{1}H NMR). Here, I analyzed exudates of the two

selected fungi with a focus on oxalate. In **chapter 6**, I confirmed that both fungi release the compound into the M9+propionate medium in a concentration range of 0.075–0.093 % [w/v], as evidenced by the HPLC analyses of the exudates. The fungal exudates also contained other compounds such as glycerol, formic acid, acetic acid, fumaric acid and citric acid, in addition to other unidentified compounds. This further strengthened the tenet that the two fungi in soil (and possibly others as well) attract bacteria via the release of oxalate. It is important to note that many other chemical molecules are also likely to have an effect in the BFI, but these were not addressed, with respect to their potential function, in this thesis.

Overall analysis of the data and conclusions

On the basis of the data described in this thesis, I posit that key genetic systems / traits that might act as the potential cornerstones of *B. terrae* –fungal interactions in soil are multifold, and potentially act in concert. The speculative list, with a brief description of the evidence supporting this tenet, is shown in Table 7.1. Also, the questions posed in the introduction to this thesis, with the answers obtained through experimentation, are shown in Fig. 7.1.

Briefly, from the work presented in this thesis, the following conclusions are drawn:

1. The 'mycosphere' presents a particular niche to soil bacteria, providing them with resources that guarantee their survival. Inhabitants of the mycosphere, such as *B. terrae*, are endowed with genetic traits, including (integrated) plasmids, that enable them to explore the available niches.
2. The genome of the fungal–interactive *B. terrae* BS001 carries a suite of genetic traits that confer the capacity to interact with the soil fungus *Lyophyllum* sp. strain Karsten.
3. HGT events have shaped the genome of *B. terrae* BS001 over evolutionary time, as evidenced by the occurrence of a suite of RGPs. Such RGPs are hypothesized to enhance the strain BS001 ecological fitness required for (a) its successful survival in soil microhabitats and (b) its interactivity with soil organisms, including (but not confined to) soil fungi.
4. *B. terrae* BS001 expresses several genes differentially during its interaction with *Lyophyllum* sp. strain Karsten. Genetic systems involved in chemotaxis towards the growing fungal hyphae, metabolism and stress responses are positively involved. Moreover, some stress resulting from the local conditions was alleviated by the fungus.
5. *B. terrae* BS001 moves chemotactically towards two selected fungi on SEA plates and on M9 agar. The chemotactic responses towards these fungi in the presence of extra added glycerol are amplified. However, with respect to oxalate, the behaviour of strain BS001 towards fungi follows a pattern of being vigorous at a lower concentration and lethargic at higher concentrations.

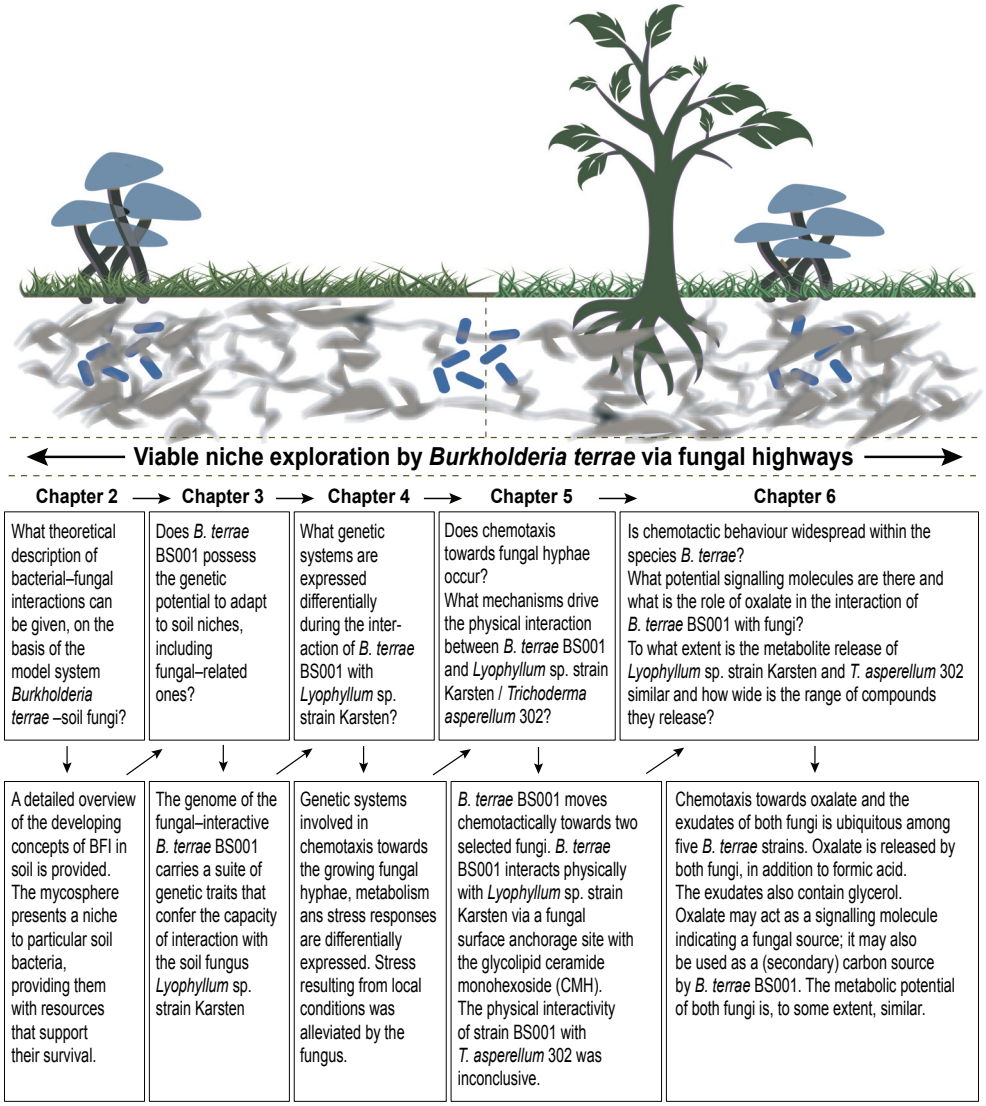

Figure 7.1 A schematic flow chart of thesis questions and the results. Graphic resources for this figure were obtained from www.freepik.com, available as free vectors and used after modifications.

6. *B. terrae* BS001 physically interacts with *Lyophyllum* sp. strain Karsten via a fungal surface anchorage site with the glycolipid ceramide monohexosides (CMH). It is at present unknown to what extent this interaction is exclusive.
7. The metabolites released by *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 are to some extent similar and so relatively similar conditions are shaped in both mycospheres.
8. The capacity of chemotactic movement towards oxalate and the exudates of both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 is omnipresent across the *B. terrae* strains BS001, BS110, BS007, BS437 and type strain 17804^T.
9. Oxalate is released by both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, in addition to formic acid, citric acid, acetic acid and fumaric acid. These exudates also contain glycerol.
10. Oxalate or oxalic acid act as positive signalling molecules. These may also be used as a (secondary) carbon source by *B. terrae* BS001.
11. All *B. terrae* strains analyzed in this thesis possess the necessary genetic potential to utilize and degrade oxalate and its complexes.

Outlook – future perspectives

Although this thesis answered some of the outstanding research questions pertaining to the ecology and mechanisms governing the BFI involving *B. terrae* in soil, using *in vitro* studies, answers to some of the complex questions still remain at large. Thus future work should be focused on the exploration of the secondary metabolite production potential, and its regulation by ecological conditions, of both *B. terrae* strain BS001 and the associated fungi. Such an endeavor will open up new avenues that will assist in a better understanding of the *B. terrae* – fungal interactions. One may hypothesize that the production and release of such molecules has large ecological significance, potentially modulating the growth and/or development of the interaction partner. Or, of other (neighboring) organisms in the same sphere. Such studies also bear the enormous potential of finding new antibiotics, as evident from the work of others, i.e. the interaction of *B. rhizoxinica* HKI454 and *Rhizopus microsporus* (Partida–Martinez and Hertweck 2005).

In **chapter 4** I found glimpses of something unique that might play a role in the interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten. It involved the upregulation of a so–called 'Suppressor of variegation–Enhancer of zeste–Trithorax' (SET) domain containing protein during the interaction. The role of SET domain containing proteins in bacterial–fungal interactions is not yet understood. However, in the light of the available literature, I surmised that such proteins may be used by the bacterium to, indeed, modulate the physiological status of the fungus. Might this modulation be at the basis of the inhibition of primordium setting, as described previously by Nazir et al. (2013)? In order to understand the exact role of the upregulated SET domain protein, a mutation–based approach is recommended. In general, I advocate the use of hypothesis–driven approaches like this one, see also Table 7.1 for more prospective confirmatory trajectories.

This thesis also yielded data pointing at the strong upregulation of a dedicated cluster of five genes in the presence of the fungus. Although a role of this cluster in the utilization of oxalate and the concomitant detoxification of reactive oxygen species is a possibility, I here did not elaborate further on the clarification of such a potential mechanism. Clearly, there is room to further explore the role of the gene cluster in the interaction, including its timing, again via hypothesis–driven approaches (and possibly using mutagenesis). Given that the five–gene cluster was present across all the tested *B. terrae* strains and is flanked by repeats and transposases (unpublished data), there is a possibility of it having been acquired horizontally in the past. Evolutionary theory then predicts it to have conferred ecological fitness to these strains, potentially in the mycosphere and beyond. In the light of this arguments, a dedicated focus should be placed on the likely origin of such gene clusters.

Further explorations of the *B. terrae* – fungal interactome should also include an examination of the dynamics and relevance of oxalate in the mycosphere and its potential role in BFI. Even though I did find some answers to the questions regarding its role as a signalling molecule and potential carbon source, a better clarification of the involvement of any or all of the oxalate degradation systems is required. Finally, in the light of the presence of glycerol, oxalate, fumaric acid, acetic acid, formic acid and fumarate in the mycosphere, a better focus should be placed on the putative diauxic growth patterns of *B. terrae* BS001 in the mycosphere, when confronted with such compounds as sources of carbon and energy.

References

Ben-Jacob E, Finkelshtein A, Ariel G, Ingham C. 2016. Multispecies swarms of social microorganisms as moving ecosystems. Trends in Microbiology, 24:257–269.

Chet I, Mitchell R. 1976. Ecological aspects of microbial chemotactic behavior. Annual Review of Microbiology, 30:221–239.

De Boer W, Folman LB, Summerbell RC, Boddy L. 2005. Living in a fungal world: Impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews, 29:795–811.

Dijkhuizen L, Wiersma M, Harder W. 1977. Energy production and growth of *Pseudomonas oxalaticus* OX1 on oxalate and formate. Archives of Microbiology, 115:229–236.

Dijkhuizen U, Knight M, Harder W. 1978. Metabolic regulation in *Pseudomonas oxalaticus* OX1. Autotrophic and heterotrophic growth on mixed substrates. Archives of Microbiology, 116:77–83.

Estrada-de los Santos PP, Vinuesa L, Martínez-Aguilar, Hirsch AM, Caballero-Mellado J. 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. Current Microbiology 67:51–60.

Finlay RD. 2007. The fungi in soil. In J. D. van Elsas, J. K. Jansson, J. T. Trevors, (Eds.), Modern soil microbiology. (pp. 107–146). New York: CRC Press.

Kohlmeier S, Smits TH, Ford RM, Keel C, Harms H, Wick LY. 2005. Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi. Environmental Sciences and Technology, 39:4640–4646.

Konstantinidis KT, Tiedje JM. 2004. Trends between gene content and genome size in prokaryotic species with larger genomes. Proceedings of the National Academy of Sciences of the United States of America, 101:3160–3165.

Kost T, Stopnisek N, Agnoli K, Eberl L, Weisskopf L. 2014. Oxalotrophy, a widespread trait of plant-associated *Burkholderia* species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*. Frontiers in Microbiology, 4:421.

Leibold MA, Holyoak M, Mouquet N, Amarasekare P, Chase JM, Hoopes MF, Holt RD, Shurin JB, Law R, Tilman D, Loreau M, Gonzalez A. 2004. The metacommunity concept: a frame-work for multi-scale community ecology. Ecology Letters, 7:601–613.

Nazir R, Tazetdinova DI, van Elsas JD. 2014. *Burkholderia terrae* BS001 migrates proficiently with diverse fungal hosts through soil and provides protection from antifungal agents. Frontiers in Microbiology, 5:598.

Nazir R, Warmink JA, Voordes DC, van de Bovenkamp HH, van Elsas JD. 2013. Inhibition of mushroom formation and induction of glycerol release–ecological strategies of *Burkholderia terrae* BS001 to create a hospitable niche at the fungus *Lyophyllum* sp. strain Karsten. Microbial Ecology, 65:245–254.

Nazir R, Zhang MZ, de Boer W, van Elsas JD. 2012. The capacity to comigrate with *Lyophyllum* sp. strain Karsten through different soils is spread among several phylogenetic groups within the genus *Burkholderia*. Soil Biology and Biochemistry, 50:221–233.

Partida-Martinez LP, Groth I, Schmitt I, Richter W, Roth M, Hertweck C. 2007. *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. Int. J. Syst. Evol. Microbiol. 57:2583–2590.

Partida-Martinez LP, Hertweck C. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature, 437:884–888.

Rudnick MB, van Veen JA, de Boer W. 2015. Oxalic acid: a signal molecule for fungus-feeding bacteria of the genus *Collimonas*? Environmental Microbiology Reports, 7:709–714.

Schneider K, Skovran E, Vorholt JA. 2012. Oxalyl-coenzyme A reduction to glyoxylate is the preferred route of oxalate assimilation in *Methylobacterium extorquens* AM1. Journal of Bacteriology, 194:3144–3155.

Standing D, Killham K. 2007. The soil environment. In J. D. van Elsas, J. K. Jansson, J. T. Trevors, (Eds.), Modern soil microbiology. (pp. 1–22). New York: CRC Press.

Stopnisek N, Zühlke D, Carlier A, Barberán A, Fierer N, Becher D, Riedel K, Eberl L, Weisskopf L. 2015. Molecular mechanisms underlying the close association between soil *Burkholderia* and fungi. The ISME Journal, 10:253–264.

Warmink JA, van Elsas JD. 2008. Selection of bacterial populations in the mycosphere of *Laccaria proxima*: Is type III secretion involved? The ISME Journal, 2:887–900.

Warmink JA, van Elsas JD. 2009. Migratory response of soil bacteria to *Lyophyllum* sp. strain Karsten in soil microcosms. Applied and Environmental Microbiology, 75:2820–2830.

Yang HC, Im WT, Kim KK, An DS, Lee ST. 2006. *Burkholderia terrae* sp. nov., isolated from a forest soil. International Journal of Systematic and Evolutionary Microbiology, 56:453–457.

Zentmyer GA. 1961. Chemotaxis of zoospores for root exudates. Science, 133:1595–1596.